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(57) Abstract

Disclosed is a method for the treatment of a patient infected with an intracellular pathogen involving administering to the patient a sub-sample of the patient's own PBMCs enriched for pathogen-specific cytotoxic T lymphocytes. Such a CTL sub-sample is prepared by (i) isolating a cell from the mammal; (ii) expressing in the cell a nucleic acid which encodes a pathogen-specific polypeptide, whereby the polypeptide is processed by the cell and an antigenic portion of the polypeptide is presented on the cell surface; and (iii) contacting this antigen-presenting cell with a sample of the mammal's peripheral blood mononuclear cells (PBMCs) to stimulate the production of the enriched sub-sample of CTLs which recognize and which are capable of lysing pathogen-infected cells of the mammal.

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PATHOGEN-SPECIFIC CTL THERAPY

Background Of The Invention

This invention relates to therapies involving administration of cytotoxic T lymphocytes (or cells).

Cytotoxic T cells (CTLs) that specifically lyse HIV-1 infected autologous target cells have been found to occur at uncommonly high frequency in the blood of HIV-infected individuals; killing by such cells is predominantly mediated by $CD3^+CD8^+$ effector cells although cytotoxic $CD4^+$ cells and natural killer cells also play a role (Walker et al., *Nature* 328:345, 1987; Plata et al., *Nature* 328:348, 1987; Walker et al., *Science* 240:64, 1988; Sethi et al., *Nature* 335:178, 1988; Koenig et al., *Proc. Natl. Acad. Sci. USA* 85:8638, 1988; Nixon et al., *Nature* 336:484, 1988; Tsubota et al., *J. Exp. Med.* 619:1421, 1989; Riviere et al., *J. Virol.* 63:2270, 1989; Koup et al., *Blood* 73:1909, 1989; Hoffenbach et al., *J. Immunol.* 142:452, 1989; Culmann et al., *Eur. J. Immunol.* 19:2383, 1989; and Hosmalin et al., *Proc. Natl. Acad. Sci. USA* 87:2344, 1990). $CD8^+$ T cells recognize antigenic peptides presented by MHC class I molecules. To be recognized by a CTL, a peptide must be properly processed, be capable of binding to MHC strongly enough to compete with other peptides, and be recognized as a peptide-MHC complex by T cells in the repertoire. Recent studies indicate that in some infections only a small number of peptides meet these criteria and that CTL specific for these epitopes dominate the lytic response (Braciale et al., *Immunol. Rev.* 98:95, 1987; Whitton et al., *J. Virol.* 62:687, 1988; Klavinskis et al., *J. Virol.* 63:4311, 1989; Whitton et al.,

J. Virol. 62:687, 1988; Braciale et al., *Proc. Natl. Acad. Sci. USA* 86:277, 1989; Townsend et al., *Cell* 44:959, 1986).

Summary Of The Invention

In general, the invention features a method of treating a mammal infected with an intracellular pathogen. The method involves (i) isolating a cell from the mammal; (ii) expressing in the cell a purified nucleic acid which encodes a pathogen-specific polypeptide, whereby the polypeptide is processed by the cell and an antigenic portion of the polypeptide is presented on the surface of the cell; (iii) contacting the antigen-presenting cell with a sample of the mammal's peripheral blood mononuclear cells (PBMCs) to produce a sub-sample enriched for cytotoxic T lymphocytes which recognize and which are capable of lysing the pathogen-infected cells of the mammal; and (iv) administering to the mammal an infection-reducing amount of the cytotoxic T lymphocyte sub-sample.

In various preferred embodiments, the antigen-presenting cell is an autologous peripheral blood mononuclear cell, preferably an autologous monocyte or an autologous B cell; the nucleic acid is expressed from a vaccinia vector or a Bacillus Calmette-Guerin vector; the intracellular pathogen is a virus (for example, an immunodeficiency virus, a T cell leukemia virus, a Herpes virus, or an Epstein-Barr virus), a mycobacterium, a protozoan, a mycoplasma, or a fungus; and the mammal is a human. Most preferably, the virus is a human immunodeficiency virus, and the nucleic acid encodes a human immunodeficiency virus *env*, *gag*, or *pol* protein.

By "intracellular pathogen" is meant a disease-causing organism which resides, during at least a part of its life cycle, within a host cell. By "enriched for

cytotoxic T lymphocytes" is meant that the sub-sample has a substantially greater number of pathogen-specific cytotoxic T lymphocytes (i.e., T lymphocytes which recognize and destroy cells bearing foreign antigens, in this case, pathogen-specific antigens, on their surfaces) than a freshly isolated sample of the patient's peripheral blood mononuclear cells. By "a purified nucleic acid" is meant a nucleic acid which encodes (as used herein) the pathogen-specific polypeptide (and which may also include upstream or downstream regulatory elements) but which is free of the genes that, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene encoding the pathogen-specific polypeptide. By "pathogen-specific polypeptide" is meant any chain of amino acids which includes an antigen specific to the pathogen; as used herein, such an antigen is recognized (i.e., responded to as foreign) by the cells, in this case, cytotoxic T cells, of the patient's immune system. By "processed" is meant converted into an antigenic peptide (e.g., by proteolysis or denaturation) and displayed on the cell's surface in association with an MHC class I antigen. By "lyse" is meant to destroy or disintegrate, for example, a host cell harboring a pathogen. By "pathogen-infected cells" is meant those host cells harboring a pathogen, either in an active or a latent state. By "antigen-presenting cell" is meant any cell capable of displaying on its cell surface an antigen, or an immunogenic fragment thereof. By "autologous" is meant occurring in the same patient. By "immunodeficiency virus" is meant, without limitation, HIV-1 and HIV-2; by "T cell leukemia virus" is meant, without limitation, HTLV-I and HTLV-II; by "Herpes virus" is meant, without limitation, Herpes simplex type 1 and type 2, Herpes zoster, and cytomegalovirus as well as

Epstein-Barr virus. By "virus" is also meant, without limitation, Papillomavirus, Hepatitis virus, Creutzfeldt-Jakob virus, and feline leukemia virus. By "mycobacterium" is meant, without limitation, Mycobacterium leprae or Mycobacterium tuberculosis. By "protozoan" is meant, without limitation, Toxoplasma gondii, Giardia lamblia, Trypanosoma cruzi, organisms of the genus Leishmania, and organisms of the genus Plasmodium which cause malaria. By fungus is meant, without limitation, Pneumocystis carinii, Candida albicans, and Candida tropicalis.

In the method of the present invention, the CTLs of the enriched sub-sample recognize and selectively target for lysis pathogen-infected cells. Because such pathogen-infected cells represent a small percentage of the total cell population, this method minimizes side effects, such as immunosuppression, which may result from other forms of therapy such as those which destroy or impair the function of all host cells at risk of pathogen infection. Moreover, the pathogen-specific CTL population may be administered to the mammal free of (or with a low dose of) lymphokines, thereby avoiding the vascular-leak syndrome which generally accompanies the superphysiologic doses of lymphokines associated with such therapies, at least in humans and mice. Finally, the CTL sub-sample is produced by contact with an antigen-presenting cell derived from the same mammal. This is an important feature of the invention because antigens capable of inducing an effective CTL response (i.e., inducing significant CTL proliferation) may vary, and, for example, in the case of HIV-1, do vary from one individual to the next. By allowing an individual's own cells to process the pathogen-specific polypeptide, the antigen(s) which are immunogenic (and, very likely, immunodominant) for that particular individual are naturally selected and

presented on the cell surface. When this antigen-presenting cell is used for the preparation of pathogen-specific CTLs, such CTLs target infected host cells which display that same immunogenic or immunodominant antigen on their surface, thereby maximizing the efficacy of the therapy.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawings will first briefly be described.

Drawings

FIG. 1 is a graph showing HIV-1-specific cytotoxicity as a function of the effector:target cell ratio. The effector cells recognize an HIV-1 envelope antigen.

FIG. 2 is a graph showing HIV-1-specific cytotoxicity as a function of the effector:target cell ratio. The effector cells recognize an HIV-1 envelope antigen.

FIG. 3 is a graph showing HIV-1-specific cytotoxicity as a function of the effector:target cell ratio. The effector cells recognize an HIV-1 reverse transcriptase antigen.

FIG. 4A is a graph showing HIV-1-specific cytotoxicity as a function of effector:target ratio before stimulation with an autologous HIV-1 antigen presenting cell. FIG. 4B is a bar graph showing that effector cells recognize a gag-specific antigen following stimulation.

FIG. 5 is a series of three graphs showing HIV-1-specific cytotoxicity as a function of effector:target ratio. The effector cells (from a single HIV-1-infected subject) recognize an HIV-1 envelope antigen (A), an HIV-1

reverse transcriptase antigen (B), and an HIV-1 gag antigen (C).

There now follow descriptions of methods for generating, from a sample of a patient's peripheral blood mononuclear cells, a sub-sample enriched in cytotoxic T lymphocytes (CTL) which recognize and lyse cells displaying an HIV-1-specific antigen on their cell surfaces. The method generally involves introducing into a patient's cells (specifically, the patient's peripheral blood mononuclear cells) a vector expressing all or a part of one or several HIV-1 protein(s). The protein is processed by the patient's cells, and an antigenic fragment is naturally presented on the cell surface. Such cells are then used as antigen-presenting cells to stimulate the proliferation of an HIV-1-specific sample of cytotoxic T lymphocytes.

T-Lymphocyte Culture

In a first specific example, a frozen aliquot of $3x10^6$ peripheral blood mononuclear cells (PBMC), isolated by Ficoll-Hypaque density gradient centrifugation from heparinized blood, was defrosted, suspended in T cell medium [i.e., RPMI 1640 medium supplemented with 15% fetal calf serum (JRH Biosciences, Lenexa, KS), 200 U/ml rhu IL2 (Cetus, Norwalk, CT), 2 mM glutamine, 2mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol] and allowed to adhere at room temperature to two wells of a 24-well microtiter plate. Following a four-hour incubation, non-adherent cells were removed and placed at 4°C overnight. Adherent cells were infected at approximately 2 pfu/adherent cell with either vPE16 (a recombinant vaccinia vector which expresses the HIV-1_{BH8} gp160 envelope protein; available from the National Institute of Allergy and Infectious Disease AIDS Depository,

Bethesda, MD) or vSC8 (a recombinant vaccinia vector which expresses the lacZ protein; available from the National Institute of Allergy and Infectious Disease AIDS Depository, Bethesda, MD). Cells were incubated with rocking, at 37°C over CO_2 for 30 minutes, an additional 1 ml of media was added, and the cells were further incubated overnight. Following this incubation, the supernatant from the infected cells was aspirated, and the vaccinia virus was inactivated by 30 minutes of UV-irradiation and 5000 rad of γ-irradiation. The reserved non-adherent cell sample was then added to the infected adherent cells (in 2 ml/well), and the cells were incubated for 10 days and fed biweekly with the T cell medium described above.

The non-adherent cell layer was then restimulated using autologous B cells as antigen-presenting cells (APCs). These APCs were prepared as follows. 10^7 PBMCs were suspended in 0.5 ml of RPMI medium supplemented with 10% fetal calf serum and incubated in a 25 cm^2 flask at 37°C, with mixing, for one hour. To these cultured cells was first added 0.5 ml of an Epstein-Barr virus-containing supernatant obtained from the marmoset cell line B95-8 (available from the American Type Culture Collection, Rockville, MD; ATCC Accession No. CRL 1612) and then added 500 μl of a 1μg/ml solution of cyclosporin and 3.5 ml of the B cell medium [i.e., RPMI 1640 medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine, 2mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol]. The cells were then incubated, with biweekly changes of medium, until they enlarged, formed clumps, and divided at an exponential rate. At this point, the cells were considered to be immortalized (see, e.g., Blumberg et al., *J. Infect. Dis.* 155:877, 1987)

and were infected overnight with 2 pfu/cell of either vSC8 or vPE16 (as described above). The vaccinia recombinant virus vector was inactivated by 30 minutes of UV-irradiation and 5000 rad of γ-irradiation.

To stimulate CTL proliferation, $5x10^5$ antigen-presenting autologous B cells were added to each well of non-adherent cells and, following an additional one week incubation, cytolytic activity of this CTL sub-sample was assayed against lacZ-expressing or HIV-1 envelope-expressing targets by the standard ^{51}Chromium-release assay described in Brunner et al. (*Immunology* 14:181, 1968).

HIV-vaccinia-infected autologous B cells were used as target cells at an effector:target (E:T) ratio of 20:1. The results shown in Table 1 were obtained. Results are expressed as percent specific cytotoxicity and were calculated from the average cpm as [(average cpm - spontaneous release)/(total release - spontaneous release)] x 100.

TABLE 1

T Cell Effector	Uninfected Autologous B Cell Target	LacZ-Expressing B-Cell Target	Env-Expressing B-Cell Target
Unselected	12	Not done	11
Selected with LacZ control	Not done	6	Not done
Selected with Env	Not done	17	39

As shown in Table 1, unselected T cells did not demonstrate any envelope-specific cytotoxicity at an E:T ratio of 20:1. In contrast, at the same E:T ratio, envelope-selected T cells lysed envelope-expressing target

cells 22% more efficiently than they lysed lacZ-expressing target cells (control). In addition, CTL sub-samples prepared by the above method specifically target and lyse Epstein-Barr virus-infected host cells. Such CTL sub-samples are particularly useful for the treatment of patients doubly-infected with a human immunodeficiency virus and an Epstein-Barr virus, who are at risk of developing B cell lymphomas.

In a second specific example, peripheral blood mononuclear cells were freshly isolated from a patient with AIDS-defining constitutional symptoms. The PBMC (at $5x10^5$ cells/well in a 24 well microtiter plate) were infected with 2 pfu/cell of vSC8 (described above), vPE16 (described above), or vDK1 (a recombinant vaccinia vector which expresses HIV-1_{HXB2} gag protein; available from the AIDS Research and Reference Program, Bethesda, MD). Following an overnight incubation in the T cell medium (as described above), the supernatant from the infected cells was aspirated, and the infected cells were subjected to 30 minutes of UV-irradiation and 5000 rad of γ-irradiation. $2x10^6$ freshly isolated PBMC were then added to each well in the same medium. The medium in the wells was replaced biweekly and, after 17 days, the gag-stimulated line was restimulated with autologous B cells that were infected with 2 pfu/ml vDK1 (as described above). Twelve days post-stimulation, a 4 hour ^{51}Cr-release assay was performed to assess envelope-specific or gag-specific cytotoxicity. Experiments were carried out using a series of effector:target ratios, and results were expressed as percent specific cytotoxicity (as described above).

TABLE 2

T Cell Effector	Protein Expressed By Target Cell	Effector:Target Ratio 6:1	12:1	25:1	50:1
Unselected	LacZ	4	6	3	0
	Env	3	6	8	14
	Gag	0	0	7	11
Gag-selected	LacZ	0	Not done	Not done	Not done
	Env	6	Not done	Not done	Not done
	Gag	18	Not done	Not done	Not done

As shown in Table 2, the nonspecifically-stimulated T cells (i.e., as derived from the patient) had a low level of cytotoxicity directed against gag-expressing autologous target cells. (This line also exhibited a low level of cytotoxicity against reverse transcriptase-expressing autologous target cells). The gag-selected T cells developed substantial cytotoxicity against gag-expressing target cells. At an E:T ratio of 6:1, cytotoxicity against gag-expressing target cells was comparable to that measured for unselected T cells at an E:T ratio of 50:1. Envelope-stimulated and lacZ-stimulated T cells did not grow well and were not assayed.

Using the above assay, five HIV-infected patients were tested for specific T cell stimulation.

Figs. 1 and 2 demonstrate that HIV-1-specific CTLs were produced from the PBMCs of two independent HIV-1-infected individuals following stimulation with autologous envelope-expressing cells (i.e., autologous B cells which were infected with vaccinia vector vPE16, described above). The percent specific cytotoxicity exhibited by envelope-

selected CTLs (—•—) was greater than that exhibited by an unselected (- - •- -) cell line. Background cytotoxicity was determined using lacZ-expressing target cells (—o—).

Fig. 3 demonstrates that an HIV-1-specific T cell line may be generated from the PBMCs of an HIV-1-infected individual following stimulation with autologous reverse transcriptase-expressing cells (i.e., autologous B cells which were infected with vaccinia vector vCF21; available from the National Institute of Allergy and Infectious Disease AIDS Depository, Bethesda, MD). Again, the percent specific cytotoxicity exhibited by the reverse transcriptase-selected CTLs (—•—) was greater than that exhibited by an unselected (- - •- -) cell line. Background cytotoxicity was assayed using lacZ-expressing target cells (—o—).

Fig. 4 demonstrates that an HIV-1-specific T cell line may be generated from the PBMCs of an HIV-1-infected individual following stimulation with autologous gag-expressing cells (i.e., autologous B cells which were infected with vaccinia vector vDK1; available from the AIDS Research and Reference Program, Bethesda, MD). This patient had no significant HIV-specific cytotoxicity before selection (Fig. 4A), and, again, the percent specific cytotoxicity exhibited by the gag-selected CTLs ("vDK1") was greater than that exhibited by the unselected ("vSC8") cell line (Fig. 4B).

Fig. 5 demonstrates that different HIV-1-specific T cell lines may be generated from the PBMCs of a single HIV-1-infected individual following stimulation with either autologous envelope-expressing cells (Fig. 5A), autologous reverse-transcriptase-expressing cells (Fig. 5B), or autologous gag-expressing cells (Fig. 5C) (specifically, by selecting the T cell line against autologous B cells

infected with either vaccinia vector vPE16, vCF21, or vDK1, respectively). Each of the T cell lines was tested for cytotoxicity against target cells expressing an envelope antigen from vPE16 (—◆—), a reverse transcriptase antigen from vCF21 (—◇—), a gag antigen from vDK1 (—■—), or a background lacZ antigen control from vSC8 (—□—). In all cases, the percent specific cytotoxicity exhibited by the HIV-1 antigen-selected CTLs was greater than that exhibited by an unselected cell line.

In an alternative preferred method for generating an enriched pathogen-specific CTL sub-sample, a T cell line is first established by incubating 5 X 10^5 previously frozen or freshly isolated PBMCs (isolated as described above) in T cell medium supplemented with phytohemagglutinin [i.e., RPMI 1640 medium supplemented with 15% fetal calf serum (JRH Biosciences, Lenexa, KS), 200 U/ml rhu IL2 (Cetus, Norwalk, CT), 2 mM glutamine, 2mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol, and 2μg/ml phytohemagglutinin (PHA-P; Difco)]. Twice a week, cultures are adjusted to 5 X 10^5 cells/ml, and after (preferably) three weeks, this T cell line is incubated with antigen-presenting cells (as described above) to enrich for pathogen-specific CTLs. If desired, T cells may be frozen for storage after 7-10 days of culture (as described above) by aliquoting 2 X 10^7 cells/vial in 95% calf serum/5% DMSO and freezing in liquid nitrogen using a programmed cell freezer (Cryomed). Such an extended culture system may also be used to expand a CTL sub-sample following pathogen-specific CTL selection.

In the examples described above, antigen-presenting cells were derived from either total peripheral blood

mononuclear cells, the adherent layer of a sample of peripheral blood mononuclear cells, or autologous B cells.

Therapy

Sub-samples enriched for pathogen-specific CTLs are administered to a pathogen-infected patient as follows. Cells are washed twice with PBS to remove culture medium, resuspended in 200 ml of the same medium, and infused back into the patient by the standard techniques developed for cancer therapy by Rosenberg (see, e.g., Rosenberg et al., *N. Eng. J. Med.* 319:1676, 1988). Typically, infusion is performed intravenously using 10^7 - 10^{11} cells, and the procedure takes approximately 30 minutes. If necessary, treatment can be repeated, preferably at 2-3 week intervals. Therapy can be administered soon after pathogen infection or upon onset of symptoms. In addition, one or more PBMC samples isolated from a pathogen-infected, asymptomatic individual, or a CTL-enriched sub-sample prepared following pathogen infection, may be stored, frozen in liquid nitrogen (as described above), until such time as that patient requires therapy.

Because the CTLs of the enriched sub-sample recognize and selectively target pathogen-infected cells and because such pathogen-infected cells represent a small percentage of the total cell population, this method minimizes side effects resulting from generalized cell damage. In the specific example of an HIV-infected patient, the enriched CTL sub-sample would target HIV-infected CD4 lymphocytes, monocytes and macrophages, leaving other cells of the immune system (including uninfected CD4-bearing lymphocytic and monocytic cells) intact and thus reducing the risk of immunosuppression. This method also avoids the

side effects, e.g., the vascular-leak syndrome associated with high dosage lymphokine therapy.

When appropriate, lymphokines such as IL-2 or IL-4 may be co-administered with a sub-sample of pathogen-specific CTL-enriched lymphocytes to further enhance lymphocyte proliferation. To minimize the side effects often associated with this treatment, a patient may be treated with antihistamines, aspirin, or acetaminophen prior to administration of lymphokines. In addition, a patient may be treated with cyclophosphamide or other cytotoxic drugs prior to administration of pathogen-specific CTL-stimulatory peptides or pathogen-specific CTLs.

Pathogen-specific CTLs can combat pathogen infection by recognizing and lysing cells infected with the pathogen, thereby preventing further spread of infection. Moreover, certain pathogen-specific CTLs, e.g., CTLs specific for Epstein-Barr virus, can be used to prevent or to treat a virus-induced lymphoma in a patient infected with EBV alone or in a patient infected with EBV and a human immunodeficiency virus.

Other Embodiments

The general methods described herein may be modified, e.g., to simplify production of the antigen-presenting cells, to simplify production of the pathogen-specific CTL sub-sample, or to extend the use of the method to the treatment of other pathogenic infections.

For example, other vaccinia vectors may be utilized for antigen expression. Such vectors include vPE8 (a vaccinia-HIV-1_{BH10} gp120 vector), vPE5 (a vaccinia-HIV-1_{BH8} gp160 vector), vPE6 (a vaccinia- HIV-1_{BH8} gp120 vector), vSC40 (a vaccinia-HIV-1_{BH10} gag-pol vector), vCF21 (a

vaccinia-HIV-1_{HXB2} reverse transcriptase vector), vVK1 (a vaccinia-HIV-1_{HXB2} gag-pol vector), VV:gag (a vaccinia-HIV-1_{HXB} gag vector), vMN462 (a vaccinia-HIV-1_{MN} gp160 vector), vRF222 (a vaccinia-HIV-1_{RF} gp160 vector), pIII*env*3-1 (a pSV2-*dhfr*-HIV-1_{HXB2} rev and gp160 vector), pDOLHIV*env* (a pDOL-HIV-1 gp160, tat, and rev vector), pH*env* (a pIII*env*3-1 HIV-1 gp160, tat, and rev vector), pNL4-3d*Pst* (a pUC18-HIV-1_{LAV} gag and pol vector); all available from the AIDS Research and Reference Reagent Program (Bethesda, MD). Other particular vaccinia vectors include vPE17, vPE18, vPE20, vPE21, and vPE22 (available from the National Institute of Allergy and Infectious Disease AIDS Depository, Bethesda, MD) which encode truncated envelope proteins and vCF32, vCF33, vCF34, vCF35, vCF36, and vCF37 (available from National Institute of Allergy and Infectious Disease AIDS Depository, Bethesda, MD) which encode truncated reverse transcriptase proteins. Methods for constructing other vaccinia expression vectors are provided, e.g., in Chakrabarti et al., *Nature* 320:535, 1986; Flexner et al., *Virology* 166:339, 1988; Earl et al., *Proc. Natl. Acad. Sci. USA* 87:648, 1990; Earl et al., *J. Virol.* 65:31, 1991; and Mackett et al. in *DNA Cloning: A Practical Approach*, Vol. II, ed., D. Glover, IRL Press, Oxford.

In addition, because all cells express MHC class I surface antigens, any cell may act as an antigen-presenting cell. Cells of the immune system (e.g., monocytes, macrophages, B cells, or T cells) are preferred. However, other useful cells include fibroblasts or neurons. In addition, cells infected with Epstein Barr virus (as described above) represent preferred antigen presenting cells.

Appropriate vectors for antigen expression in these (and other) cell types are provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1989); preferred vectors include a poxvirus, avipox virus, SV40, retrovirus, Epstein-Barr virus, or bovine papilloma virus sequence. An HIV mutant, for example, a packaging or frameshift mutant, may also be used to infect an appropriate antigen-presenting cell for HIV-specific CTL enrichment (see, e.g., Aldovini and Young, *J. Virol.* 64:1920, 1990).

In one particular example, hepatocytes infected with an antigen-expressing hepatitis B vector may be used as an antigen-presenting cell. In other particular examples, peripheral blood lymphocytes are transduced with an antigen-presenting retroviral vector (e.g., T4-pMV7, T8-MV7, or pHIV*lacZ*; available from the AIDS Research and Reference Reagent Program, Bethesda, MD).

Expression vectors may be introduced by stable or transient transfection or by viral infection. Transfection methods are provided, e.g., in *Current Protocols in Molecular Biology* (ed. Ausubel et al., John Wiley & Sons, New York, 1989); viral infection methods are generally as provided above. When necessary, expression vectors (e.g., viral expression vectors) may be inactivated by UV-irradiation (as described above), γ-irradiation (as described above), exposure to mutagens (e.g., mitomycin C), treatment with glutaraldehyde, or inactivation of a gene(s) required for infectivity (e.g., by site directed mutagenesis or deletion).

CTL-stimulatory antigens may be included in HIV-1-encoded proteins other than env (i.e., gp160) and gag. For example, they may be included in the reverse transcriptase, tat, rev, or nef proteins. DNA encoding such proteins or

fragments thereof may be inserted into a vaccinia expression vector (or any appropriate expression vector as defined above), and the resultant antigen-presenting cells used to produce an enriched sub-sample of HIV-1-specific CTLs as described in the above example. In particular examples, antigen-presenting cells may be prepared by infection of cells (preferably, monocytes, macrophages, T cells, or B cells) with one of the following vectors: vPE8, vPE5, vPE6, vSC40, vVK1, VV:gag, vMN462, vRF222, pIII*env*3-1, pDOLHIV*env*, pH*env*, or pNL4-3d*Pst* (all described above); or pCV1 (a pCV-HIV-1 tat and rev vector), pSV2tat72 (a pSV2-*dhfr*-HIV-1 tat vector), or pU3R-III CAT (a pSV2CAT-HIV-1 LTR vector) (all available from the AIDS Research and Reference Reagent Program, Bethesda, MD). Any isolate of HIV may be used as a source of viral-specific polypeptide genes (including virus isolated from an HIV-infected patient's lymphocytes). Moreover, a patient infected with any isolate of HIV (e.g., HIV-1_{MN}) may be treated using the methods of the invention. Genes coding for proteins homologous to HIV-1-encoded proteins may also be useful in this invention if such proteins elicit an HIV-1-specific CTL response; the proteins are generally coded for by related primate lentiviruses, e.g., HTLV-I and HTLV-II as well as the simian immunodeficiency viruses. Less preferably, the antigen-presenting cell may be infected with HIV itself (e.g., virus isolated from the subject to be treated), although the level of antigen expression is generally lower than that obtained using expression vectors such as those described herein.

The instant methods can be used to treat human patients or mammals infected with other pathogenic viruses including, but not limited to, human T-cell leukemia viruses, Herpes viruses (e.g., Epstein-Barr virus), and

Hepatitis virus as well as any intracellular disease-causing mycobacterium, mycoplasma, protozoan, or fungus. In one particular example, antigen-presenting cells may be prepared by infection of cells (preferably, monocytes, macrophages, T cells, or B cells) with one of the following HIV-2 vectors: vSC50 (a vaccinia-HIV-$22_{SBL/ISY}$ gp160 vector), rVV/ROD (a vaccinia-HIV-2_{ROD} gp160 vector), or rVV/ST (a vaccinia-HIV-2_{ST} gp160 vector); all available from the AIDS Research and Reference Program (Bethesda, MD). In another particular example, the mycobacterium Bacillus Calmette-Guerin (BCG) may be engineered to encode foreign proteins and used to infect, e.g., monocytes or macrophages, to produce antigen-presenting cells; such mycobacterium and methods for infection are described in Aldovini and Young (*Nature* 351:479, 1991). Generally, the sub-samples of pathogen-specific CTLs would be prepared as described above for HIV-1.

The PBMC sample may be presented simultaneously with more than one pathogen-specific CTL-stimulatory epitope, e.g., by expressing more than one pathogen-specific gene in the antigen-presenting cell or by contacting the PBMC sample with multiple APCs, each displaying a different pathogen-specific antigen. In a preferred CTL selection procedure, a sample of PBMCs or a T cell line is presented with two or more different autologous antigen presenting cells at the same time -- for example, a first APC expressing the HIV-1 envelope gene, a second APC expressing the HIV-1 gag, and a third APC expressing the HIV-1 reverse transcriptase gene; such APCs may be produced, e.g., by infection of the autologous cell with an HIV-1-vaccinia vector (e.g., as described herein).

Any pathogen-infected mammal (particularly, domesticated animals and livestock) may be treated using a pathogen-specific CTL sub-sample of the invention.

Claims

1. A method of preparing a therapeutic agent for the treatment of a mammal, preferably a human, infected with an intracellular pathogen, said method comprising:

(i) isolating a cell from said mammal;

(ii) expressing in said cell a purified nucleic acid which encodes a pathogen-specific polypeptide, whereby said polypeptide is processed by said cell and an antigenic portion of said polypeptide is presented on the surface of said cell;

(iii) contacting said antigen-presenting cell with a sample of said mammal's peripheral blood mononuclear cells (PBMCs) to produce a sub-sample enriched for cytotoxic T lymphocytes which recognize and which are capable of lysing said pathogen-infected cells of said mammal; and

(iv) providing said cytotoxic T lymphocyte sub-sample in a dosage form providing an infection-reducing amount thereof .

2. The method of claim 1, wherein said antigen-presenting cell is an autologous peripheral blood mononuclear cell.

3. The method of claim 2 wherein said antigen-presenting cell is an autologous monocyte.

4 The method of claim 2 wherein said antigen-presenting cell is an autologous B cell.

5. The method of claim 1, wherein nucleic acid is expressed from a vaccinia vector.

6. The method of claim 1, wherein nucleic acid is expressed from a Bacillus Calmette-Guerin vector.

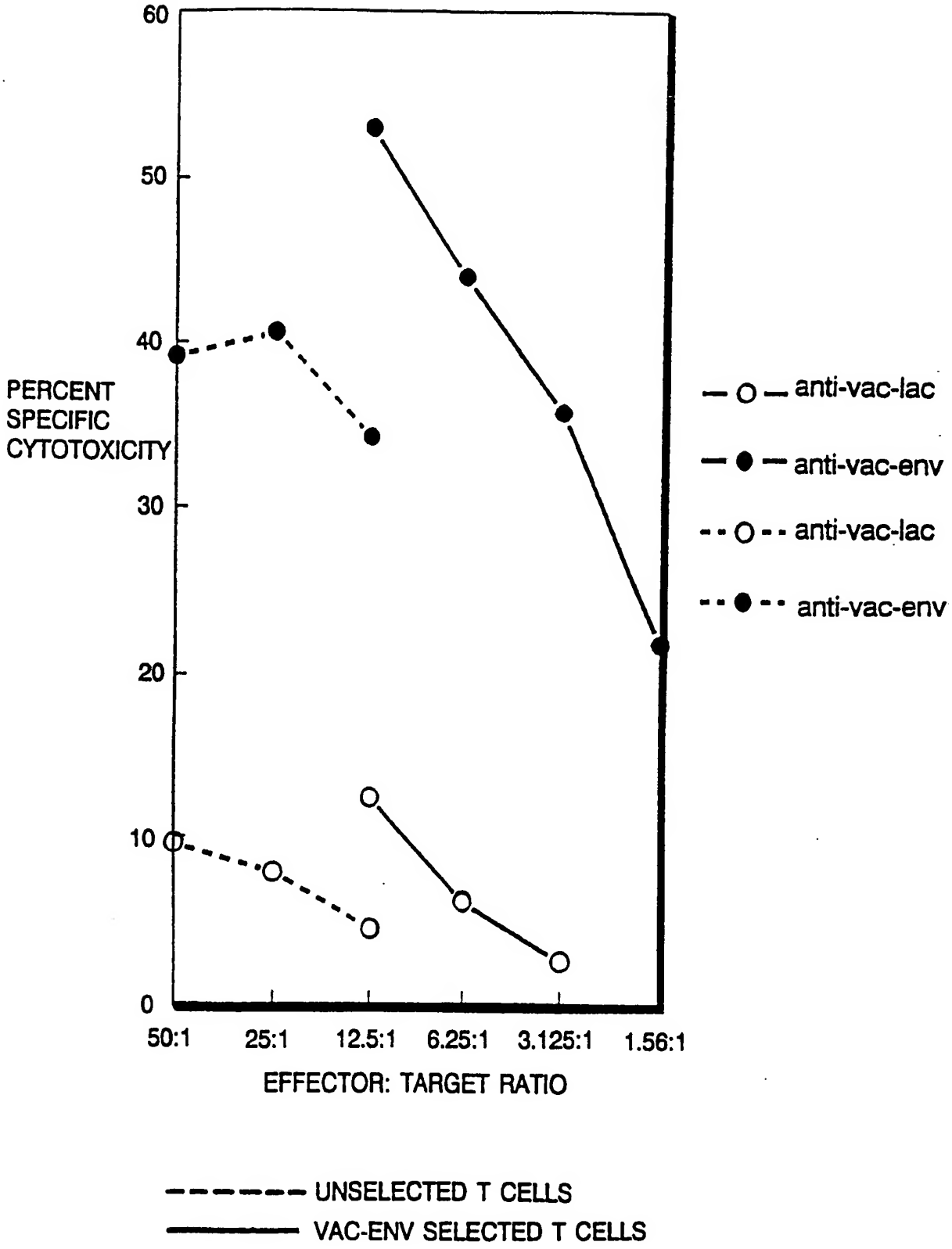
7. The method of claim 1, wherein said intracellular pathogen is a virus, a mycobacterium, a protozoan, a mycoplasma, or a fungus.

8. The method of claim 7, wherein said virus is an immunodeficiency virus.

9. The method of claim 8, wherein said nucleic acid encodes a human immunodeficiency virus env protein, a human immunodeficiency virus gag protein, or a human immunodeficiency virus pol protein.

10. The method if claim 7, wherein said virus is a T cell leukemia virus.

11. The method of claim 7, wherein said virus is a Herpes virus, preferably an Epstein-Barr virus.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6.25:1
3.125:1
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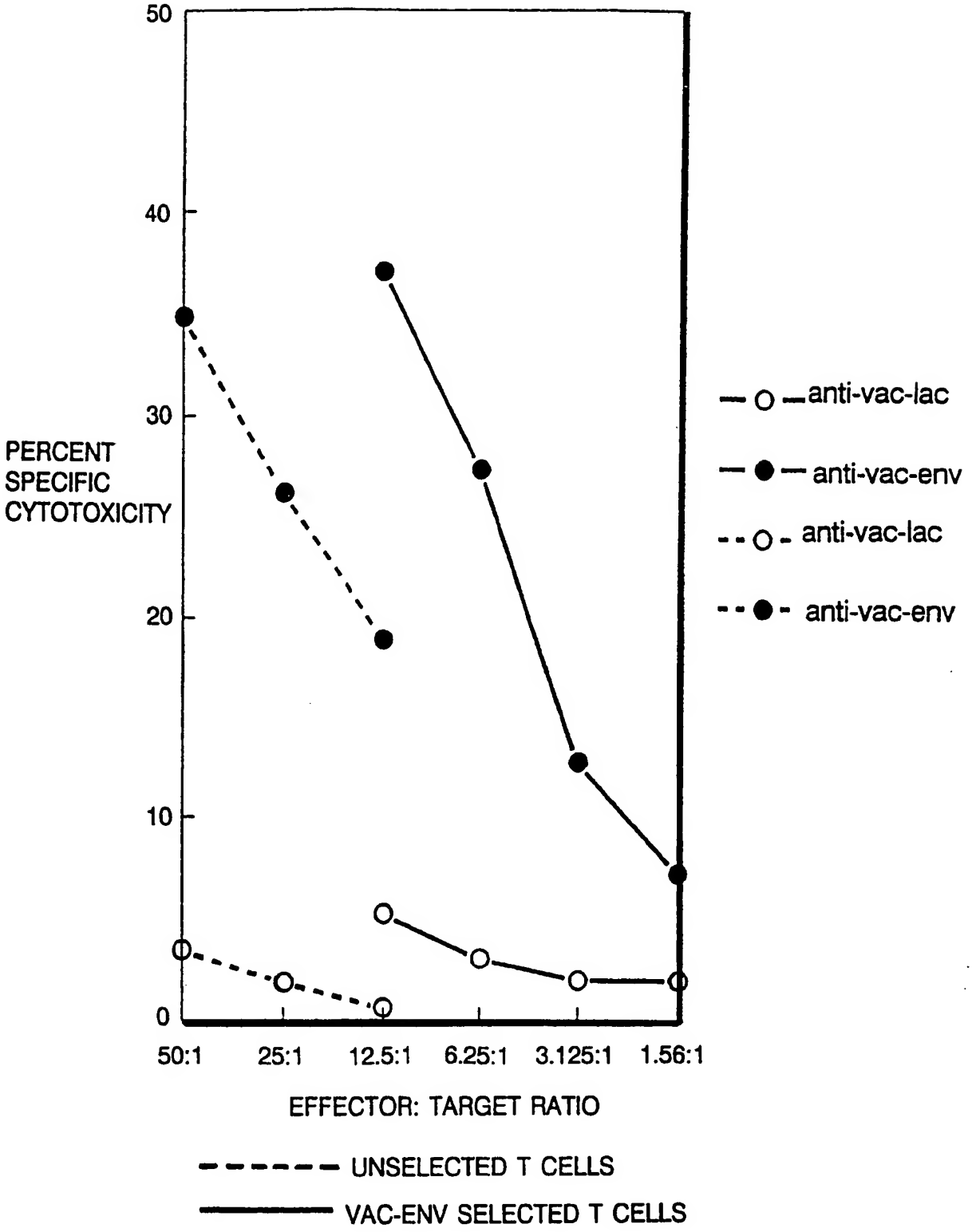
anti-vac-env
anti-vac-lac
anti-vac-env
UNSELECTED T CELLS
VAC-ENV SELECTED T CELLS

FIG. 1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12.5:1
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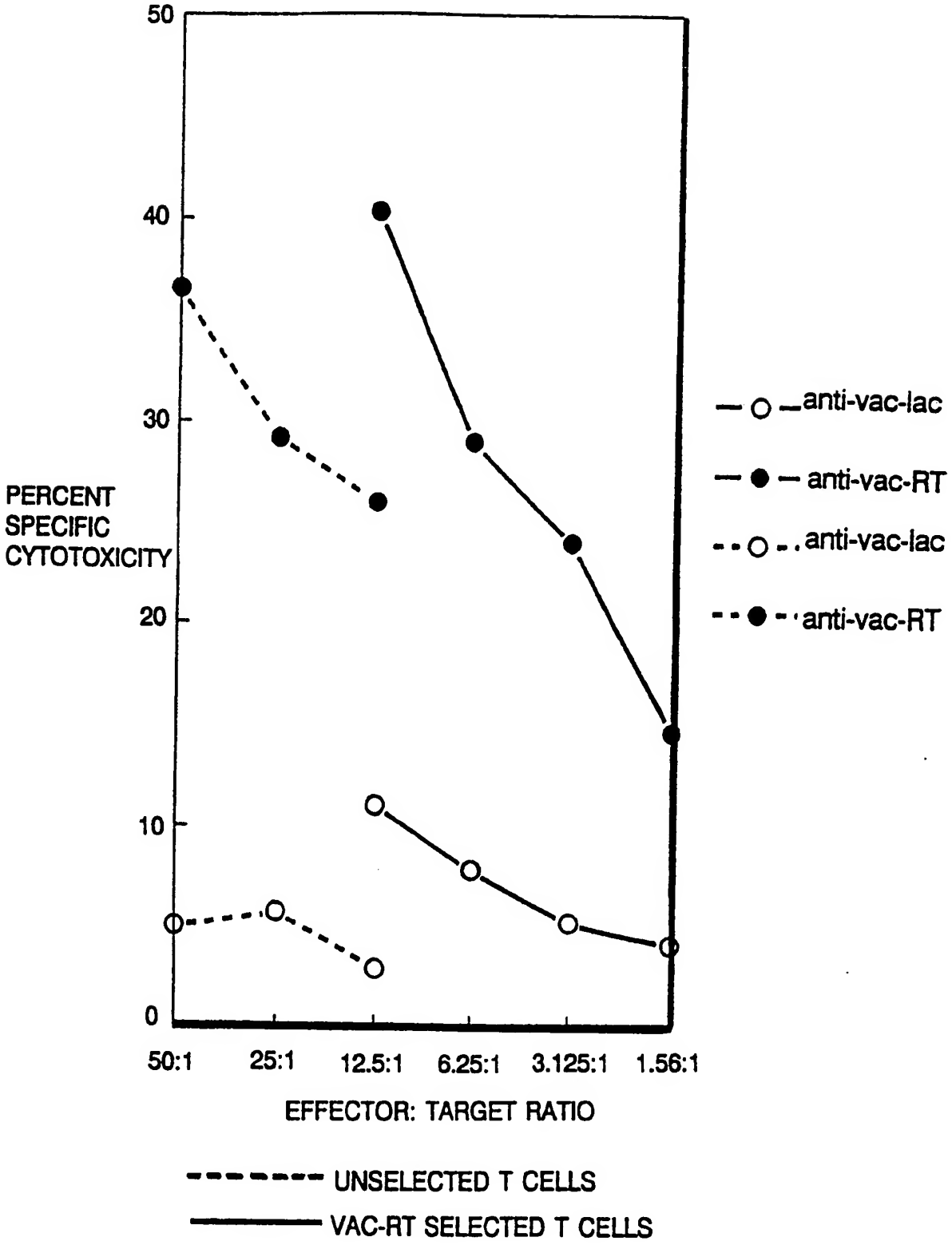
UNSELECTED T CELLS
VAC-ENV SELECTED T CELLS

FIG. 2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1.56:1
EFFECTOR: TARGET RATIO
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anti-vac-RT
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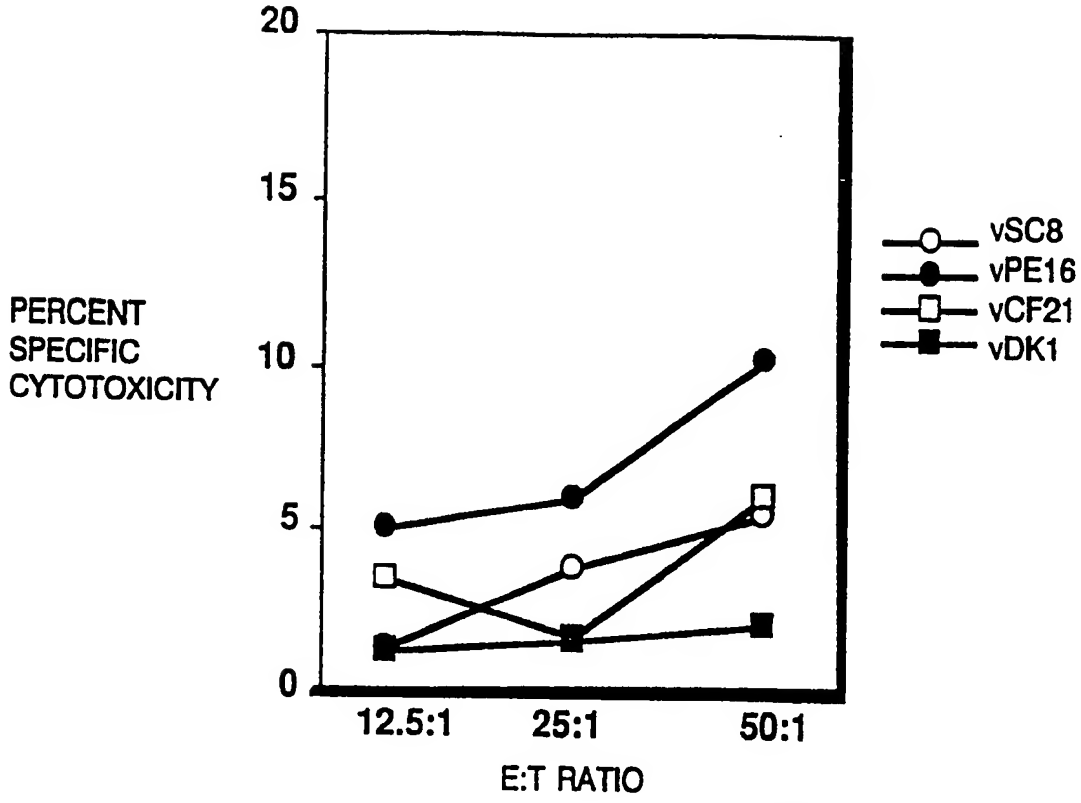
VAC-RT SELECTED T CELLS

FIG. 3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vPE16
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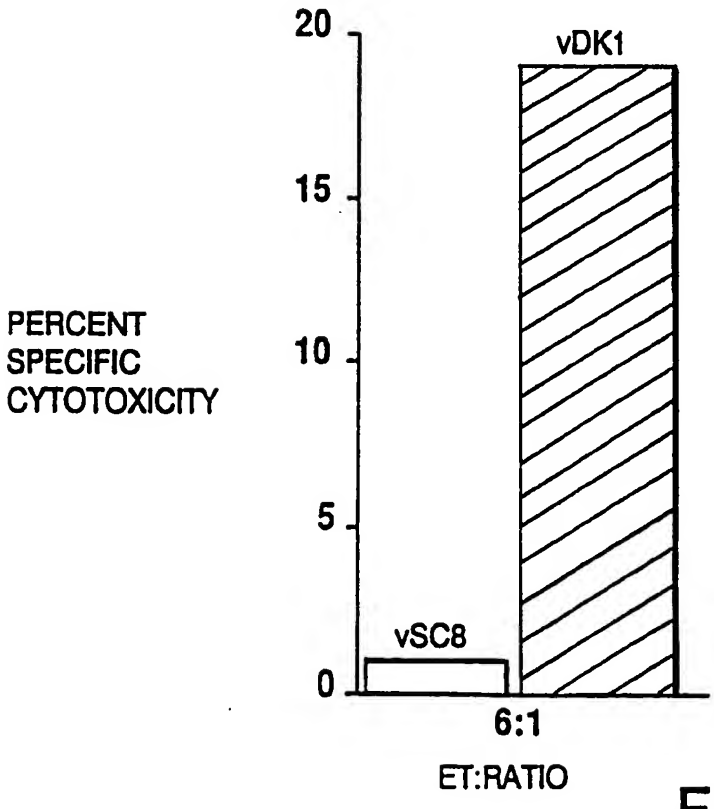
FIG. 4a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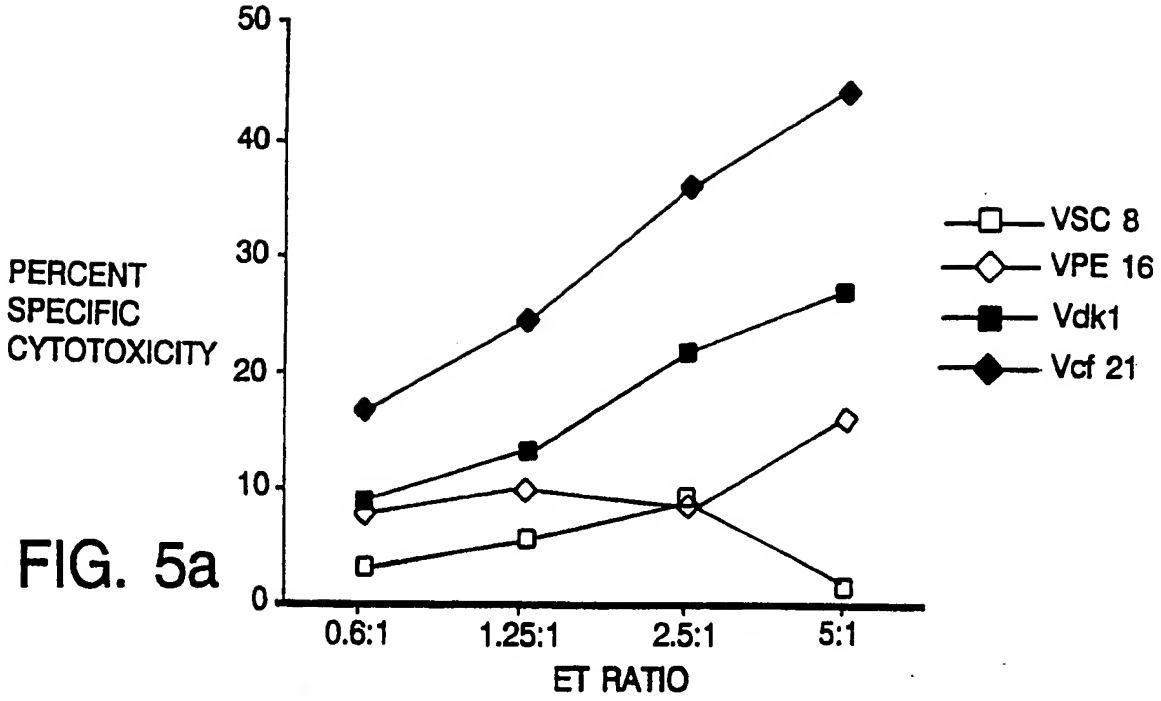
ET:RATIO

FIG. 4b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ET RATIO
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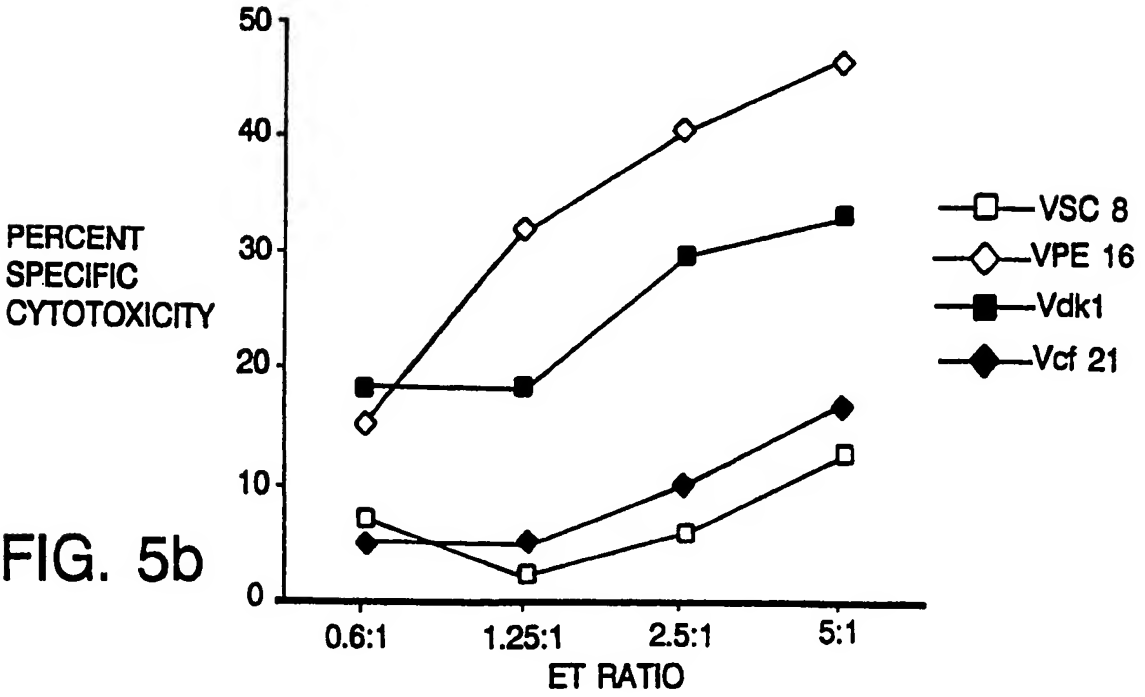
VPE 16
Vdk1
Vcf 21

FIG. 5a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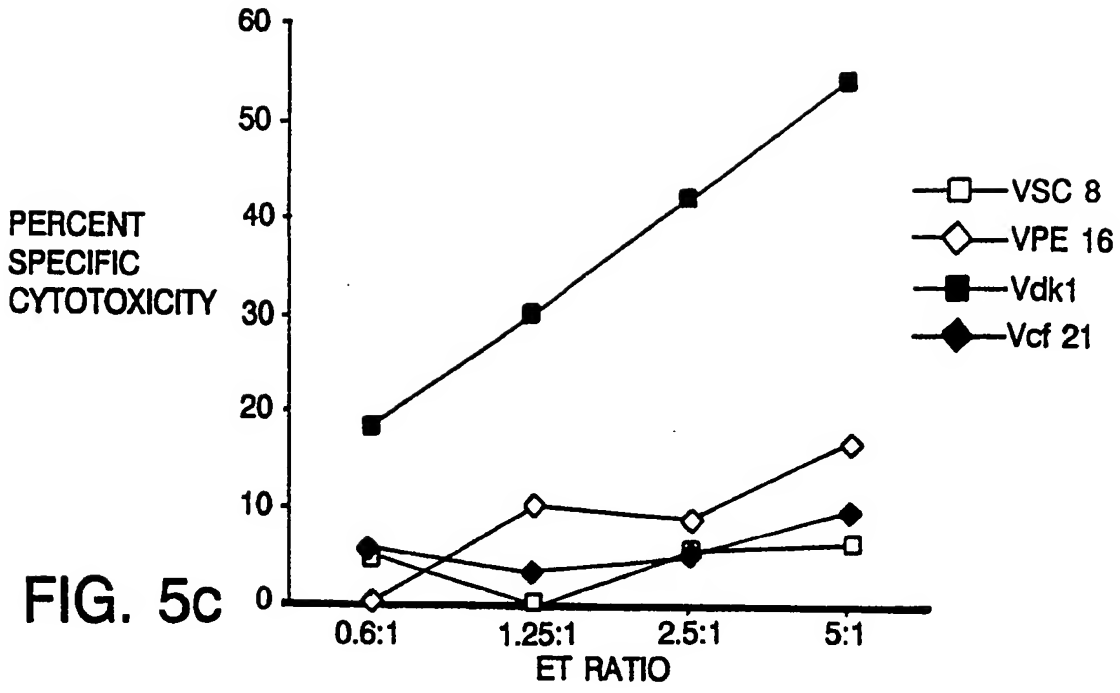
ET RATIO
VSC 8
VPE 16
Vdk1
Vcf 21

FIG. 5b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SPECIFIC
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1.25:1
2.5:1
5:1
ET RATIO
VSC 8
VPE 16
Vdk1
Vcf 21

FIG. 5c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00619

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 45/05, 39/21
US CL :424/93A
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1985+, AUTOMATED PATENT SEARCH (U.S.)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,081,029 (Zarling et al) 14 January 1992. See column 1, lines 50-68 through column 2, line 3. Also, see column 15, lines 50-52.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 March 1993	Date of mailing of the international search report 02 APR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JOHNNY F. RAILEY II Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*